APPENDIX C

[illegible] laminae, after administration of apomorphine, is of considerably greater interest. It is noteworthy that it is in layer VI and the deepest portion of layer V that the dopaminergic terminals[8] and dopamine-sensitive cells[9] have been identified in the rostral area of the medial aspect of the frontal cortex. There is little evidence, however, of a similar dopaminergic system in layer VI of the dorsolateral frontal cortex and the sensory motor cortex. The regions of cortex in which glucose utilisation increased after administration of apomorphine correspond closely with those areas to which the ventral nucleus of the thalamus projects[10,11]. Glucose utilisation in this thalamic nucleus was increased after administration of apomorphine, and the metabolic activation observed in the dorsolateral frontal and sensory motor cortices may reflect, at least in part, increased activity of non-dopaminergic thalamocortical systems.

The interpretation of the results of any neuropharmacological investigation depends on the specificity of action of the agents used. In the study reported here, the sensitivity to apomorphine of glucose utilisation in areas such as layer VI of the sensory motor cortex, and the prevention of the apomorphine-induced alterations in glucose utilisation in all cortical areas by low doses of the dopamine-antagonist, haloperidol, suggest a key involvement of dopaminergic receptors in the responses observed. Our finding that the cortical involvement in the action of apomorphine extends beyond the known confines of the mesocortical dopaminergic system suggests a need for reappraisal of the mechanisms and foci of action underlying the behavioural effects of antipsychotic and other drugs, which are used to manipulate dopaminergic systems in the CNS.

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Growth and differentiation of aggregating fetal brain cells in a serum-free defined medium

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Aggregating cultures of mechanically dissociated fetal brain cells provide an excellent system for neurobiological studies of cellular growth and differentiation[1-4], but, in common with many cell culture systems, they have the disadvantage that crude serum is required in the medium. Although several cell lines have either been adapted to serum-free conditions[5-7] or grown directly in serum-free media supplemented with hormones, trace elements and defined serum components[12-14], this approach has never been applied to differentiating primary cells of the central nervous system. We now describe the successful cultivation of aggregating fetal rat brain cells in a chemically defined, serum-free medium.

Fig. 1 Electron micrograph of a 35-d aggregate grown in S⁻ medium, showing a myelinated axon and a synaptic contact. The tissue was fixed in paraformaldehyde–glutaraldehyde, postfixed in osmium tetroxide and embedded in Epon. Sections were stained with uranyl acetate and lead citrate (×42,000).

Equal numbers of mechanically dissociated fetal (15–16 d gestation) rat brain cells[1-3] were inoculated either in Dulbecco–Vogt modified Eagle's medium (DMEM) containing fetal calf serum, referred to as S^+ medium, or in serum-free DMEM supplemented with insulin (5 $\mu g\ ml^{-1}$), 20 nM hydrocortisone, 0.3 nM triiodothyronine, transferrin 1 $\mu g\ ml^{-1}$ and trace elements (see Table 1 legend), referred to as S^- medium. In either case the dissociated cells re-aggregated readily to give a uniform population of aggregates. Throughout the culture period, aggregates in S^- medium remained somewhat smaller, but more numerous (two or threefold) than those in S^+ medium.

Cell proliferation was examined by measuring both the total DNA content and the incorporation of ^3H-thymidine into a trichloroacetic acid (TCA)-precipitable macromolecular fraction (Table 1). Cultures grown in S^- medium (S^- cultures) had a greater DNA content and a smaller protein/DNA ratio than cultures grown in S^+ medium (S^+ cultures). In addition, cell counts of thionine-stained semi-thin sections of aggregates revealed almost twice as many cells per unit area in S^- than did S^+ cultures. Both observations suggest slower cell growth (for example, protein acquisition) in S^- cultures. Incorporation of ^3H-thymidine (representing DNA synthesis) was demonstrated in both S^+ and S^- cultures (Table 1). During the first 10 d of culture, S^- cultures showed a higher rate of DNA synthesis, with maximum activity after about 5 d. After 10 d, DNA synthesis diminished more rapidly in S^- than in S^+ cultures and by day 20 it was only about one-third of that of S^+ cultures. Lack of either insulin or transferrin in S^- medium resulted in a reduction of both total DNA and ^3H-thymidine incorporation at day 5, whereas no difference was observed in the initial rate of ^3H-thymidine uptake between cultures grown either in the presence

or absence of insulin. These results suggest that both insulin and transferrin stimulate cell proliferation in S⁻ cultures, and this agrees with results of investigations with established cell cultures grown in serum-free conditions[12,14,15]. Lack of either hydrocortisone or triiodothyronine, two other hormones that stimulate the growth of some cell lines[16], had no apparent effect on DNA synthesis.

The morphological differentiation of the aggregates was followed by electron microscopy. Compared with S⁺ cultures, S⁻ cultures underwent generally slower maturation of neuronal processes and delayed myelin synthesis. In S⁻ cultures no synaptic profiles were found after 4 d, but they were frequent after 8 d; the first myelinated axons appeared at about day 30. After 35 d numerous synaptic contacts and many myelinated fibres were observed (Fig. 1). The compaction of myelin sheaths often appeared incomplete.

The biochemical differentiation of the cultures was studied at regular intervals by measuring the specific activities of the following neurotransmitter metabolising enzymes: choline acetyltransferase (CAT, EC 2.3.1.6), acetylcholinesterase (ACE, EC 3.1.1.7), glutamate decarboxylase (GAD, EC 4.1.1.15), aromatic L-amino acid decarboxylase (AAD, EC 4.1.1.26) and monoamine oxidase (MAO, EC 1.4.3.4). Table 2 shows that in S⁻ cultures, the specific activities of all enzymes (and the protein content) increased considerably during the first month. In contrast to S⁺ cultures, the phase of rapid increase in enzyme specific activities occurred later and no plateau was reached within 4 weeks (with the exception of MAO). Compared with S⁺ cultures, aggregates grown for 33 d in S⁻ had higher specific activities of GAD (192%), AAD (148%) and ACE (168%), but lower CAT (40%) and MAO (51%). Lack of insulin in S⁻ medium resulted in a significant reduction of both GAD activity and protein content, the reduction of GAD activity being more pronounced at early developmental stages (57% reduction at day 11 compared with 15% reduction at day 33). Lack of transferrin in S⁻ caused a progressive decrease in protein content and the specific activities of all enzymes except MAO. On the other hand, lack of hydrocortisone or triiodothyronine had no apparent effect on enzyme activities. In view of the relatively high GAD activity in S⁻ cultures and because the possible neuronal localisation of this enzyme required verification[20,21], we examined the formation from labelled γ-aminobutyric acid (GABA) from L-[U-¹⁴C]glutamate in aggregates (Fig. 2). Compared with S⁺ cultures, S⁻ cultures took up glutamate at a higher rate (Fig. 2a) and had almost two-fold greater net synthesis of GABA (Fig. 2b), the latter finding agreeing with the differences in GAD activity measured in homogenates (Table 2). The formation of GABA from labelled glutamate was measured in the presence of the carbonyl-trapping agent aminooxyacetic acid (AOAA). After 30 min of incubation in the presence of 13 μM AOAA, synthesis in both S⁺ and S⁻ aggregates of ¹⁴C-GABA was less than 5% of that in control cultures. This contrasts with studies on astrocyte cultures[21], in which 13 μM AOAA did not inhibit GABA formation. Furthermore, both GAD activity and rate of GABA synthesis were higher in aggregates than in astrocytes by at least an order of magnitude. Thus the GAD activity measured in aggregates probably represents the neuronal enzyme activity.

The accumulation of glutamine formed from labelled glutamate was considerably lower in S⁻ than in S⁺ cultures (Fig. 2). Glutamine synthetase (EC 6.3.1.2), the enzyme involved in the amidation of glutamate, has been shown to be localised in glial cells[22], suggesting that S⁺ cultures contained relatively fewer glial cells than S⁻ cultures. This is corroborated by the relatively low specific activity of MAO in S⁻ cultures. (Several observations suggest that the MAO activity of neuronal cells in culture is considerably lower than that of glia—ref. 23 and P., unpublished.) Thus, a proportionately higher number of neurones in S⁻ aggregates could fully account for the relative

Table 1 DNA synthesis in aggregating cultures of foetal rat brain cells

Condition	Days in culture	TCA precipitable radioactivity (10^{-6} × d.p.m. per flask)	Total DNA (μg per flask)	Total protein (mg per flask)	Protein/DNA
S⁻	2	1.2	126	3.0	24
	5	3.6	155	4.0	26
	10	3.2	120	6.1	50
S⁻, complete	2	2.9	316	4.0	13
	5	12.3	383	4.9	13
	10	6.0	270	7.0	26
S⁻, no insulin	5	4.0	265	3.4	13
S⁻, no transferrin	5	2.2	293	3.9	13
S⁻, no hydrocortisone	5	11.1	356	4.6	13
S⁻, no triiodothyronine	5	12.1	360	4.8	13

Brains of foetal (15–16 d gestation) Wistar rats (Madoma, Füllinsdorf) were dissected and dissociated mechanically as before[1,2]. The cells were washed three times with Puck's D_1 solution by centrifugation and centrifugation (3,700g_{av} per min), and resuspended in serum-free Dulbecco–Vogt modification of Eagle's medium (DMEM, high glucose, no pyruvate, GIBCO no. 320-1965, supplemented with: vitamin B_{12} 1.36 μg ml^{-1}, Sigma; biotin 0.007 μg ml^{-1}, Sigma; DL-α-tocopherol 10 μg ml^{-1}, Sigma; retinol 5 μg ml^{-1}, Fluka; lipoic acid 0.2 μg ml^{-1}, Sigma; linoleic acid 0.1 μg ml^{-1}, Sigma; penicillin 50 U ml^{-1}, ICN; and streptomycin sulphate 50 μg ml^{-1}, ICN). This single cell suspension of 3 × 10^7 viable cells per ml was divided into two equal portions and each diluted with 2 volumes of either S⁻ medium (DMEM containing 15% (v/v) foetal calf serum (Seromed) or S⁻ medium (DMEM without serum). The complete serum-free medium (S⁻) contained the following additional supplements: crystalline bovine insulin (5 μg ml^{-1}, Sigma) hydrocortisone-21-phosphate (20 nM, Sigma), 3,3',5-triiodo-L-thyronine (0.3 nM, Sigma), human transferrin (1 μg ml^{-1}, Sigma) and various trace elements as listed by Hutchings and Sato[13]. Samples (3.5 ml) of the cell suspensions were placed into 25-ml Dr Long flasks (Bellco) and incubated at 37 °C in an atmosphere of 10% CO_2/air humidified air under constant rotation at 70 r.p.m. (Infors gyratory shaker). After 2 d, cultures were transferred to 50-ml Dr Long flasks (Bellco) and 5-ml samples of new media were added. Media were changed (exchange of half medium) every 3 d. Within the first week of culturing, speed of rotation was increased gradually to 80 r.p.m. DNA synthesis was determined by measuring incorporation of ^{3}H-thymidine into a TCA-precipitable macromolecular fraction[19]. Cultures were incubated for 23 h in normal conditions with [Me-^{3}H]thymidine (Amersham, 46 Ci mmol^{-1} specific activity) to give a final concentration of 25 nM (1.2 Ci ml^{-1}). Controls were incubated for 23 h at 4 °C. Aggregates were then washed three times with solution D_1, homogenized in 0.6 ml of 0.05% (v/v) Triton X-100 using a glass–Teflon homogenizer and sonicated briefly (Branson model S-12, 3 × 3 s at 10 W). Samples (25–100 μl) of the homogenate were mixed with 1.5 ml of cold 10% (w/v) TCA, kept at 0 °C for 15 min and then collected by suction onto GF/A glass fibre filter disks. The filters were washed three times with 5% TCA, kept at 0 °C for 15 min and then collected by suction onto GF/A glass fibre filter disks. The filters were washed three times with 5% TCA, transferred to glass counting vials and incubated for 12 h in 0.5 ml NCS tissue solubiliser (Amersham). After neutralization of the digest with glacial acetic acid, 10 ml scintillation cocktail (toluene, containing 6 g l^{-1} of 2,5-diphenyloxazole (PPO) and 75 mg ml^{-1} 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP)) was added for liquid scintillation counting. Portions of homogenates were assayed for protein by modification of the method of Lowry *et al.*[18] and for DNA by a modification of the method of Kissane and Robins[19]. The values given are the means of at least two individual cultures (deviation < 10%). The average radioactivity found in controls (2,610 d.p.m. per mg protein) was subtracted.

Table 2 Development of specific activities of neurotransmitter metabolising enzymes in aggregating cultures of fetal rat brain cells

Condition	Days in culture	CAT	ACE	GAD	AAD	MAO	Total protein (mg per flask)
		Specific enzyme activity (pmol per min per mg protein)					
S^+	11	50	16,100	335	41	320	4.0
	22	235	39,300	770	67	890	8.8
	33	305	45,200	1,437	110	1,325	9.2
S^-, complete	11	11	8,700	376	64	203	5.4
	22	48	26,600	946	91	428	6.8
	33	122	76,100	2,167	163	680	8.8
S^-, no insulin	11	16	5,800	161	42	112	3.4
	22	53	24,600	485	66	381	3.4
	33	148	56,500	1,858	185	666	6.0
S^-, no transferrin	11	7	6,100	307	62	140	5.5
	22	6	13,900	230	40	390	3.9
	33	3	16,100	209	20	670	3.2
S^-, no hydrocortisone	11	8	8,300	330	50	212	5.7
	22	44	24,600	981	76	370	7.1
	33	119	73,600	968	131	701	6.5
S^-, no triiodothyronine	11	10	8,100	382	64	212	6.4
	22	41	25,300	869	93	392	6.7
	33	110	69,500	2,050	143	675	8.8

Fetal rat brain cell aggregates were prepared and cultivated in DMEM containing 15% (v/v) fetal calf serum (S^+) or in serum-free DMEM (S^-), as described in Table 1 legend. The methods used for homogenate preparation and enzyme assays have been described before[1]. Protein was determined by a modification of the method of Lowry et al.[17], using bovine serum albumin as a standard. The values given represent the mean of at least five individual culture flasks (s.e.m. <5%).

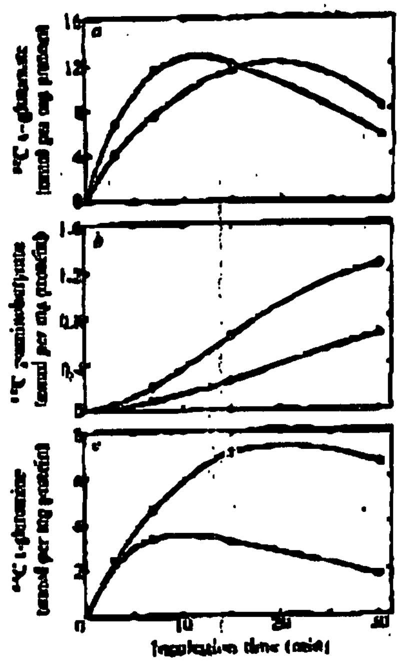

Fig. 3 Aggregates cultured for 30 d in either S^+ medium (●) or in S^- medium (○) were preincubated for 30 min in serum-free DMEM deficient in L-glutamine and subsequently incubated in the presence of 100 µM L-[U-^{14}C]glutamate (Amersham, [illegible] mCi mmol^{-1} final specific activity). The commercially available radioactive material was purified before use by TLC on cellulose [illegible]. The solvent system was 1-butanol/glacial acetic acid/water [illegible] (v/v). After various times, aggregates were analysed for their content of L-[^{14}C]glutamate (a), [^{14}C]GABA (b) and L-[^{14}C]glutamine (c). The labelled compounds were extracted from homogenates of the washed aggregates and then quantitatively separated on cellulose plates by using the following two-dimensional separation technique: high voltage electrophoresis (buffer: 0.125 M pyridine, 1.05 M acetic acid, pH 3.8), followed by TLC (solvent system as above). Further technical details are given in ref. [illegible]. Quantities of products were calculated on the assumption that the specific activity of the precursor in the tissue was equal to that in the medium. Aliquots of the homogenates were assayed for protein by a modification of the method of Lowry et al.[17] (s.e.m. < 5%).

high GAD activity observed in these cultures. If this is the case, the relatively low specific activity of CAT, another neuronal marker enzyme, may be explained if, in S^- cultures, (1) there is a smaller proportion of cholinergic neurones; (2) there is a greater retardation in the development of cholinergic neurones; or (3) CAT is more retarded in its development than GAD. Although the present results do not enable us to distinguish between these possibilities, there is some circumstantial evidence in favour of points (2) and/or (3), that is, compared with S^- cultures of cells from the mesencephalon–diencephalon–rhombencephalon region, S^- cultures of telencephalic cells (which are presumably at an earlier stage of development) show a much higher rate of DNA synthesis, a much more pronounced delay of CAT and some delay of GAD maturation (data not shown).

In conclusion, our morphological and biochemical data demonstrate that mechanically dissociated fetal rat brain cells re-aggregate, grow and differentiate in a chemically defined, serum-free medium. Such cultures show some retardation in cellular growth and differentiation compared with their counterparts grown in the presence of 15% fetal calf serum. Although more work is needed to define their developmental characteristics further, serum-free re-aggregating brain cell cultures will be valuable for the study of nutritional and hormonal influences on brain development.

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Androgen receptors exist throughout the 'critical period' of brain sexual differentiation

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Several studies suggest direct roles for androgens and oestrogens in the development of sexually dimorphic characteristics of mouse and rat brain[1]. To elucidate the biochemical mechanisms for such effects, investigators have asked which putative steroid receptors are present in the hypothalamus throughout the critical period of sexual differentiation. A priori, potential receptors would include high-affinity proteins with selectivities for active androgens, oestrogens and their metabolites. Two major classes of steroid that might be active as agonists are the androgens per se, including testosterone and dihydrotestosterone (DHT)[2], and the oestrogens, which are themselves androgen metabolites[3]. Normally, in sexual differentiation, a proper balance of both androgens and oestrogens may be necessary[4]. Indeed, receptors for each of these ligands do exist in the hypothalamus of neonatal and prepubertal mice[5-7]. Although the perinatal oestrogen receptor and its function have been extensively studied[7,8], the existence of perinatal androgen receptors has not been as clearly demonstrated to permit a similar acceptance[9]. In this report, we establish the existence of adult-like androgen receptors in embryonic and neonatal mouse and rat hypothalami by qualitative biochemical and genetic analyses. This is achieved by DNA–cellulose affinity chromatography and velocity sedimentation, and by analysis of the androgen-resistant mutant, testicular feminization. The presence of sex hormone receptors in perinatal brain is discussed in the context of behavioural responses which are differentiated during the critical period of brain sexual development.

We used DNA–cellulose affinity chromatography to characterise the androgen-binding proteins in perinatal hypothalamus as it fractionates low levels of androgen receptor in prepubertal brain[10,11] and permits qualitative analysis of the DNA-adhering material[7,8]. Figure 1 shows a representative elution profile of prepubertal hypothalamic androgen receptor (triangles), and also illustrates (circles) a ^{3}H-DHT-binding activity from neonatal hypothalamus which similarly adheres to DNA–cellulose and elutes in the same manner with a linear concentration gradient of NaCl. The androgen-binding activities in both neonatal and prepubertal cytosols exhibit elution maxima in the 140–150-mM NaCl range of the gradient. Therefore, by criteria of DNA adherence, the androgen-binding activity in perinatal mouse hypothalamus is qualitatively similar to putative androgen receptor present in older animals.

Table 1 Androgen receptors in neonatal *Tfm*/Y and sibling males

Age (d)	Genotype	Testosterone bound (mol × 10⁻¹⁸ per mg tissue)* Hypothalamic-preoptic area	Kidney
5	+/	6.7	42.0
	Tfm/Y	1.1	3.0
7	+/	7.0	50.4
	+/	7.5	48.0
	Tfm/Y	0.9	5.5

* Quantification was by DNA–cellulose affinity chromatography, using a protocol similar to that described in Fig. 1 legend.

To determine its macromolecular integrity, the material which elutes from DNA–cellulose was analysed by sucrose sedimentation (Fig. 2). As indicated by the lack of radioactivity at or near the top of sucrose gradients following centrifugation, the androgen is indeed bound to macromolecular components in cytosols from prepubertal (21 days after birth; Fig. 2a), neonatal (3–4 days after birth: Fig. 2b, closed circles) and embryonic (.. days before birth: Fig. 2b, open circles) hypothalami. The androgen-binding activities from both neonatal and embryonic hypothalami (Fig. 2b) behave similarly to that from prepubertal hypothalamus (Fig. 2a) in that all sediment as 4S macromolecules. The sedimentation profiles shown in Fig. 2a and b are representative of data obtained at all hormone concentrations tested.

Using both DNA–cellulose chromatography and velocity sedimentation, we have assessed the saturability of the androgen-binding activities in embryonic, neonatal and prepubertal tissues. Androgen-binding activities are 9[illegible] (s.e.m., n = 15) saturated at hormone concentrations of 10 nM; this is observed with both DHT and testosterone in the hypothalamic and kidney cytosols, and agrees with previous observations that putative androgen receptor from prepubertal animals saturates at hormone concentrations between 4 and 8 nM (refs 4, 7).

In mature animals with the androgen-resistant gene syndrome, testicular feminization (*Tfm*), the levels of androgen receptor are lower in both hypothalamus[4,10-13] and kidney than in wild-type animals. As the wild-type neonatal androgen-binding activity which adheres to DNA–cellulose seems to be similar to that of older animals (Figs 1, 2), we would expect this activity in neonatal *Tfm*/Y tissues to be similarly affected if the mutation is expressed at an early age. Indeed, the androgen-binding capacity of neonatal *Tfm*/Y cytosols (hypothalamus and kidney) is approximately 15% that of sibling (male and female) cytosols (Table 1): a similar observation has been made for submandibular gland and kidney cytosols using sedimentation analysis[15]. In contrast, the concentration of oestrogen receptor in mouse *Tfm*/Y hypothalamus is similar to that in sibling hypothalamus at the neonatal ages tested (data not shown) as was shown for older animals[4,10].

Thus, our data suggest that both embryonic and neonatal hypothalamus contain an androgen-binding activity which is qualitatively similar to the putative androgen receptor in the hypothalamus of older animals. However, the level of neonatal binding is lower than prepubertal binding (Figs 1, 2).

Using the qualitative DNA–cellulose and velocity sedimentation patterns presented above, we have also quantified the putative androgen receptor content of the developing mouse hypothalamus. These data are summarized in Fig. 3. The concentration of androgen receptor detected in mouse hypothalamus between embryonic and prepubertal ages increases approximately sevenfold. In contrast, between 3 days before and 3 days after birth, only a twofold increase in the concentration of androgen receptor is detected. These data augment earlier quantitative measurements which also indicate an increase between late postnatal and prepubertal ages[4,17]. Thus the phase of most rapid appearance of androgen receptor seems to coincide with the late phase of the critical period of ... sexual differentiation. This ontogeny differs significantly from that of oestrogen receptors in mouse hypothalamus, as the overall increase in the concentration of oestrogen receptor from embryonic to prepubertal ages is only twofold[8]. It is possible that technical or biological factors which differentially affect the detectability of androgen receptors cause this apparent difference. However, in spite of this qualification concerning the levels of androgen receptors, the significance of our report is that it establishes their presence in the hypothalamus throughout perinatal development.